

Initiation of Protein Synthesis in Animal Mitochondria

PURIFICATION AND CHARACTERIZATION OF TRANSLATIONAL INITIATION FACTOR 2*

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Bovine liver mitochondrial translational initiation factor 2 (IF-2_{mt}) has been purified to near homogeneity. The scheme developed results in a 24,000-fold purification of the factor with about 26% recovery of activity. SDS-polyacrylamide gel electrophoresis indicates that IF-2_{mt} has a subunit molecular mass of 85 kDa. IF-2_{mt} promotes the binding of formyl(f)Met-tRNA to mitochondrial ribosomes but is inactive with the non-formylated derivative. IF-2_{mt} is active on chloroplast 30 S ribosomal subunits, but IF-2_{chl} has no activity in promoting fMet-tRNA binding to animal mitochondrial ribosomes. IF-2_{mt} is sensitive to elevated temperatures and is inactivated by treatment with *N*-ethylmaleimide. It is partially protected from heat and *N*-ethylmaleimide inactivation by the presence of either GTP or GDP suggesting that guanine nucleotides may bind to this factor directly. The binding of fMet-tRNA to mitochondrial ribosomes requires the presence of GTP and is inhibited by GDP. DeoxyGTP is very effective in replacing GTP in promoting fMet-tRNA binding to ribosomes and some activity is also observed with ITP. No activity is observed with ATP, CTP, or UTP. Nonhydrolyzable analogs of GTP can promote formation of both 28 S and 55 S initiation complexes indicating that GTP hydrolysis is not required for subunit joining in the animal mitochondrial system.

binding and scanning mechanism for initiation such as that found in the eukaryotic cytoplasmic system (7). The small subunit of the mitochondrial ribosome appears to have the ability to bind mRNA tightly in a sequence-independent manner (8). This interaction requires an RNA having a minimal length and appears to be independent of the secondary structure of the RNA (9). It has been suggested (8) that the first step in the initiation cycle in this organelle may be the binding of the small subunit to the message.

Recently (10) the first animal mitochondrial translational initiation factor has been detected and partially purified. This factor, mitochondrial initiation factor 2 (IF-2_{mt}),¹ promotes the binding of fMet-tRNA to the mitochondrial ribosome. The binding reaction requires the presence of a message such as poly(A,U,G) and GTP. In bacterial systems the binding of the initiator tRNA to the 30 S ribosomal subunit is promoted by IF-2 in a message and GTP-dependent reaction (11). In contrast, in the eukaryotic cytoplasmic system, eIF-2 forms a ternary complex with GTP and Met-tRNA. This complex then binds to the 40 S subunit prior to the interaction of the subunit with the mRNA (12). Thus, preliminary studies suggest that IF-2_{mt} resembles the prokaryotic factor more closely than it does the eukaryotic cytoplasmic factor. In the present report, we have carried out the purification of IF-2_{mt} to near homogeneity and have examined a number of features of this factor important for its role in the initiation cycle.

EXPERIMENTAL PROCEDURES

Materials

Yeast tRNA, *Escherichia coli* tRNA^{Met}, GTP, GDP, dGTP, ITP, ATP, CTP, UTP, and poly(A,U,G) were obtained from P-L Biochemicals and Pharmacia LKB Biotechnology Inc. Phosphoenolpyruvate, pyruvate kinase and 5'-guanylimidodiphosphate (GMP-PNP) were obtained from Sigma. *E. coli* tRNA^{Met} and total yeast tRNA were from Boehringer Mannheim. Yeast tRNA^{Met} and tRNA^{Met} were separated by chromatography on BD-cellulose as described (13). [³⁵S]Methionine (1030 Ci/mmol) was obtained from DuPont-New England Nuclear. [³⁵S]fMet-tRNA and [³⁵S]Met-tRNA were prepared from yeast tRNA^{Met} and *E. coli* tRNA^{Met} as described (14). DEAE-cellulose DE52 was purchased from Whatman. High performance liquid chromatography (HPLC) columns TSKgel DEAE-5PW (preparative, 2.15 × 15 cm) and TSKgel SP-5PW (0.75 × 7.5 cm) were obtained from Beckman. Chloroplast 30 S ribosomal subunits and partially purified IF-2_{chl} were kindly supplied by Ja Seok Koo and Lan Ma (Department of Chemistry, University of North Carolina) respectively. Antiserum against *E. coli* IF-2 was a generous gift from Dr. John Hershey (Department of Biological Chemistry, University of California, Davis, CA). Partially purified *E. coli* IF-1, IF-2, and IF-3 and ribosomes were prepared as described (15).

It has been known for quite some time that animal mitochondria have their own DNA which is expressed by specific transcription and translation systems (1). Recent progress has provided a considerable amount of information on the properties of animal mitochondrial ribosomes which are 55 S particles composed of 28 S and 39 S subunits (2). In addition, three elongation factors have now been purified and partially characterized from this system (3, 4).

Much less is known about the mechanism of translational initiation in this organelle but early studies have suggested that this process will be distinct from that observed in either prokaryotes or in the eukaryotic cell cytoplasm. DNA and RNA sequence analysis (1, 5) indicates that animal mitochondrial mRNAs contain few or no nucleotides as 5' or 3' untranslated leaders. Hence, this system will not use a Shine-Dalgarno sequence type of interaction to specify the start AUG (6). Furthermore, the lack of a 5' leader or cap structure indicates that the mitochondrial ribosome will not use a cap

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¹ The abbreviations used are: IF, initiation factor; eIF, eukaryotic IF; GMP-PNP, guanyl-5'-yl-imidodiphosphate; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ITP, inosine triphosphate.

Assay for IF-2_{mt} Activity

The activity of IF-2_{mt} was determined by measuring the ability of this factor to promote the binding of fMet-tRNA to mitochondrial ribosomes using a nitrocellulose filter binding assay. Incubation mixtures contained 50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 mM spermine, 7.5 mM MgCl₂, 35 mM KCl, 0.2 mM GTP, 0.1 mM phosphoenolpyruvate, 0.1 units of pyruvate kinase, 10 µg of poly(A,U,G), 10 pmol of [³⁵S]fMet-tRNA^{Met} (20,000–80,000 cpm/pmol), mitochondrial ribosomes (0.1–0.15 A₂₆₀) and the indicated amounts of IF-2_{mt}. Reaction mixtures were analyzed as described previously (10) unless otherwise indicated. A blank representing the amount of [³⁵S]fMet-tRNA bound to mitochondrial ribosomes in the absence of IF-2_{mt} (less than 0.01 pmol) has been subtracted from each value. One unit of activity is defined as the amount of IF-2_{mt} required to promote the binding of 1 pmol of [³⁵S]fMet-tRNA to mitochondrial ribosomes under the assay conditions described.

Preparation of Bovine Liver Mitochondria and Mitochondrial Ribosomes

Digitonin-treated bovine mitochondria and mitochondrial ribosomes were prepared from 4-kg samples of fresh liver basically as described by Matthews *et al.* (16) with modifications as described (17). All steps were carried out at 4 °C or on ice. The isolated mitochondrial pellets were fast-frozen in a dry ice-isopropyl alcohol bath and stored at -70 °C until use. Mitochondrial ribosomes and a postribosomal supernatant fraction containing IF-2_{mt} were prepared as described (10). The postribosomal supernatant was dialyzed against a 10-fold excess of buffer I (20 mM Hepes-KOH, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, and 10% glycerol), fast-frozen in a dry ice-isopropyl alcohol bath, and stored at -70 °C until use.

Purification of Bovine Liver Mitochondrial IF-2

DEAE-Cellulose Chromatography—All chromatographic procedures were performed at 4 °C. The dialyzed postribosomal supernatant (800 ml containing 1800 mg of protein) prepared from about 100 g of mitochondria was subjected to chromatography on two separate DEAE-cellulose columns (90 ml, 4 × 8 cm each) equilibrated with buffer I at a flow rate of 3 ml/min. The column was washed with this buffer until the absorbance at 280 nm was less than 0.1 and was then developed with buffer I containing 0.5 M KCl. Fractions (5 ml) were collected at a flow rate of 2 ml/min. Fractions containing IF-2_{mt} activity were pooled and dialyzed against three changes of a 20-fold excess of buffer I for a total of 4 h, fast-frozen, and stored at -70 °C. The IF-2_{mt} samples from the two columns were then pooled and taken through the remainder of the purification scheme.

Preparative TSKgel DEAE-5PW—The dialyzed sample (80 ml, 340 mg) was applied to a preparative TSKgel DEAE-5PW column equilibrated with buffer I at a flow rate of 3 ml/min. The column was washed with buffer I containing 0.1 M KCl until the absorbance at 280 nm returned to base line. The column was then developed with a linear gradient (450 ml) from 0.1 to 0.25 M KCl in buffer I at a flow rate of 3.0 ml/min. Fractions (2 ml) were collected in siliconized tubes. Active fractions were pooled, dialyzed against buffer I, fast-frozen, and stored at -70 °C until use.

TSKgel SP-5PW Chromatography—The preparative TSKgel DEAE-5PW-purified sample (48 ml, 9 mg) was applied to a TSKgel SP-5PW column equilibrated with buffer I at a flow rate of 1 ml/min. The column was washed with buffer I containing 0.15 M KCl until the absorbance at 280 nm returned to base line. The column was then developed with a linear gradient (60 ml) from 0.15 to 0.33 M KCl in buffer I at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected in Eppendorf tubes. Aliquots of each fraction were removed for assay and for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The remainder of each fraction was immediately fast-frozen and stored at -70 °C until use. The aliquots of each fraction were assayed for activity, and the appropriate fractions were pooled. Purified IF-2_{mt} is very unstable and bovine serum albumin (BSA) (0.5 mg/ml) was added to the purified IF-2_{mt} preparation to improve its stability.

Sucrose Gradient Analysis of Initiation Complex Formation—Reaction mixtures (0.15 ml) were prepared as described for the IF-2_{mt} assay (10) and contained 0.42 A₂₆₀ of mitochondrial ribosomes, purified IF-2_{mt} (0.11 µg) and either GTP or the nonhydrolyzable GTP analog GMP-PNP (0.2 mM). Reaction mixtures were incubated for 20 min at 27 °C and then layered onto 4.8 ml of 10–30% (w/v) linear

sucrose gradients containing the same ionic conditions used in the initial incubation mixture. After centrifugation for 2 h at 48,000 rpm at 4 °C in a Beckman SW50.1 rotor, the gradients were displaced with a 50% sucrose solution through a flow cell with an ISCO Model 640 gradient fractionator. The absorbance profiles were continuously measured at 254 nm with an ISCO UA-5 absorbance monitor. Fractions (0.2 ml) were collected, filtered through nitrocellulose membranes, washed, and counted as described (10).

Miscellaneous Procedures

Assays on chloroplast ribosomes were carried out as described (18). Protein concentrations were determined by the method of Lowry *et al.* (19) or by the method of Sedmark and Grossberg (20) with modifications by Bearden (21) using bovine serum albumin as a standard. This procedure allows the analysis of submicrogram samples of protein. The standard curves used were based on 0.25–4 µg of protein. About 20% of the final purified sample was used for the determination of the protein concentration. SDS-PAGE was performed according to Laemmli (22) on a 7% resolving gel with a 4% stacking gel and the gel was stained with silver (23). Western blotting was carried out using a Bio-Rad Trans-Blot semidry transfer cell following the instructions recommended by the manufacturer. SDS-PAGE and Western blotting were carried out on IF-2_{mt} preparations that did not contain BSA.

RESULTS

Purification of IF-2_{mt}—Previous work (10) resulted in the identification and partial purification of IF-2_{mt}. The procedures developed in that study resulted in IF-2_{mt} preparations that were about 10% pure. We have now developed a complete purification scheme for this factor (Table I) starting from the postribosomal supernatant fraction of isolated mitochondria. In the first step of the purification procedure, the sample was subjected to chromatography on DEAE-cellulose (Table I). The recovery from this column was set at 100% for convenience since the activity of the input material cannot be measured directly (10). We estimate that there is about a 5-fold increase in specific activity as a result of this step. The sample at this stage is quite stable and can be stored for at least several months at -70 °C without significant loss of the activity.

IF-2_{mt} was further purified by HPLC column chromatography. In the first step of this procedure, the sample was applied to a preparative TSKgel DEAE-5PW HPLC column (Fig. 1A). This procedure resulted in approximately a 30-fold purification of IF-2_{mt} activity with a 79% recovery of activity (Table I). Finally, the sample was purified by chromatography on a TSKgel SP-5PW column (Fig. 1B). Two peaks of IF-2_{mt} activity were obtained from this column. The majority of the activity was present in the first peak and accounted for about 90% of the activity recovered. This material was used for the studies on the properties of this factor described below. In this step the IF-2_{mt} activity was well separated from the other proteins in the sample resulting in a 150-fold purification with a 33% recovery of activity (Table I). The overall purification procedure resulted in about a 24,000-fold purification of IF-2_{mt} from the mitochondrial postribosomal supernatant with about 26% of the initial IF-2_{mt} activity recovered.

Analysis of the last two steps in the purification scheme by SDS-PAGE indicated that the final step promoted the removal of substantial amounts of contaminating protein from the partially purified sample (Fig. 2A, lanes 1 and 2). The IF-2_{mt} sample has been purified to near homogeneity (Fig. 2A, lane 2) and this factor appears to be composed of a single polypeptide species having a molecular mass of about 85 kDa. SDS-PAGE analysis of each fraction from the TSKgel SP-5PW (Fig. 2B) indicated that the IF-2_{mt} activity in each fraction correlated with the intensity of the 85-kDa protein band. Fractions from both the major peak and the minor peak

TABLE I
Purification of IF-2_{mt}

Step	Protein mg	Activity units	Specific activity units/mg	Overall recovery %	Estimated purification -fold
Postribosomal supernatant ^a	1,800				
DEAE-cellulose	340	380	1	100 ^b	5
TSKgel DEAE-5PW	9.2	300	33	79	170
TSKgel SP-5PW	0.02	98	4,900	26	24,000

^a IF-2_{mt} activity cannot be measured in the postribosomal supernatant.

^b This value set at 100% since input activity cannot be measured directly. The estimated fold-purification in this step is indicated as 5-fold based on the amount of protein recovered.

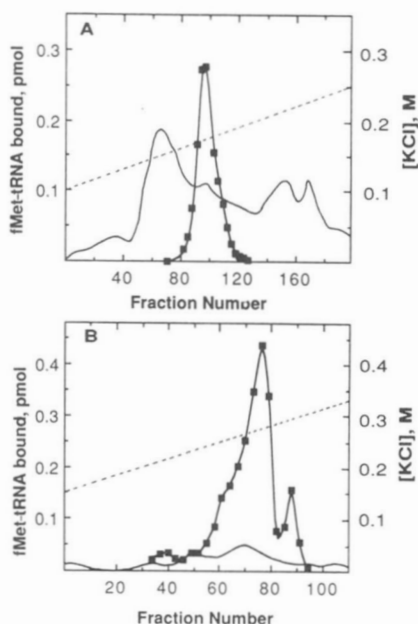


FIG. 1. Elution profiles of IF-2_{mt} on TSKgel DEAE-5PW preparative HPLC and TSKgel SP-5PW HPLC. A, IF-2_{mt} initially purified by gravity DEAE-cellulose chromatography was subjected to chromatography on a preparative TSKgel DEAE-5PW column as described under "Experimental Procedures." Aliquots (30 μ l) of various fractions were tested for IF-2_{mt} activity (■). The absorbance at 280 nm was monitored (solid line) with an ISCO UA-5 absorbance monitor on a 0.5 scale and the column was developed with a salt gradient (dashed line). B, the preparative TSKgel DEAE-5PW column-purified sample was then subjected to chromatography on a TSKgel SP-5PW as described under "Experimental Procedures." Aliquots (30 μ l) of various fractions were tested for IF-2_{mt} activity (■). The absorbance at 280 nm was monitored (solid line) with an ISCO UA-5 absorbance monitor on a 0.05 scale and the column was developed with a salt gradient (dashed line).

of IF-2_{mt} appeared to contain the same polypeptide. These results suggest that the IF-2_{mt} present in both the major and minor peaks are very similar. The difference in the elution pattern may be the result of a minor sequence difference or of a posttranslational modification.

The purified IF-2_{mt} was relatively stable for at least several months when stored at -70°C . However, it was very labile and would only survive for a few hours at 4°C . For this reason, fractions collected from the final purification column were immediately fast-frozen and stored at -70°C until use. Protein concentrations of greater than 0.1 mg/ml substantially stabilized IF-2_{mt} (data not shown). Thus, the purified IF-2_{mt} used for the characterization experiments described below contained 0.5 mg/ml BSA.

Physical Properties of IF-2_{mt}—Purified IF-2_{mt} has an apparent subunit molecular weight of 85,000 as determined by SDS-PAGE (Fig. 2). This value does not correspond well to

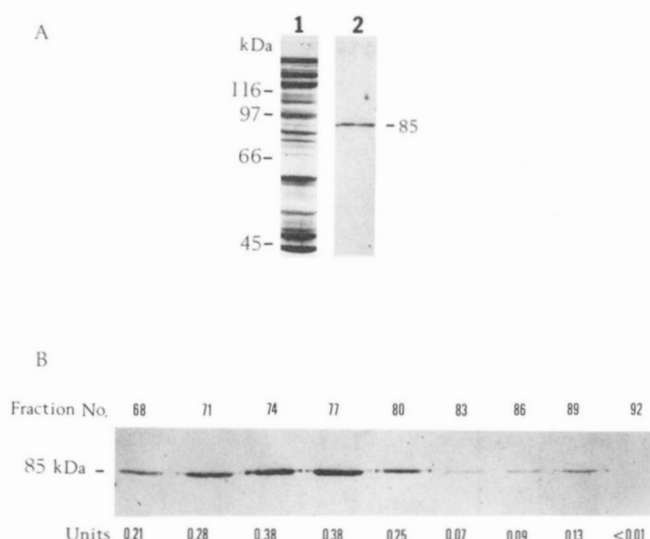


FIG. 2. SDS-PAGE analysis of IF-2_{mt}. A, lane 1, Sample from the preparative TSKgel DEAE-5PW column (2.5 μ g); lane 2, purified IF-2_{mt} (0.05 μ g) from the main peak off the TSKgel SP-5PW column. B, analysis of the activity of IF-2_{mt} and the 85-kDa band in fractions from the TSKgel SP-5PW column. Aliquots from various fractions (30 μ l) were tested for activity shown below the indicated lanes and aliquots (30 μ l) were analyzed by SDS-PAGE.

the molecular weight of 140,000 determined by gel filtration chromatography previously reported (10). In order to assess more accurately the molecular weight of IF-2_{mt}, we subjected both purified and partially purified IF-2_{mt} to the gel filtration chromatography on columns having better resolution than those used previously. Chromatography of purified IF-2_{mt} on Sephacryl-S200 resulted in a very broad distribution of activity centering at a molecular weight of about 80,000 (data not shown). The pattern of activity showed the same distribution as the 85-kDa protein band analyzed by SDS-PAGE (data not shown). This observation suggests that the purified factor is present as a monomer. However, when DEAE-cellulose preparations of this factor were subjected to chromatography on Sephacryl S-200 or Sephacryl S-300, IF-2_{mt} always eluted as a symmetrical peak with an apparent weight of about 230,000 (data not shown). This observation suggests that (in the crude sample) IF-2_{mt} maybe present as a dimer or maybe complexed with other proteins. Attempts to cross-link IF-2_{mt} in order to examine its subunit structure in solution have not yet been successful.

As reported previously (10), partially purified IF-2_{mt} is active in promoting fMet-tRNA binding to *E. coli* ribosomes suggesting that the mitochondrial factor has certain features that are conserved between the prokaryotic and organellar systems. In an effort to determine whether there is a significant homology between *E. coli* IF-2 and IF-2_{mt}, Western blots of IF-2_{mt} preparations were probed with antibodies raised

against *E. coli* IF-2. These antibodies showed no apparent cross-reaction with the mitochondrial factor (data not shown). These results indicate that IF-2_{mt} may have significant structural differences from prokaryotic IF-2.

Interaction of IF-2_{mt} with tRNA, Nucleotides, and Ribosomes—We have examined whether IF-2_{mt} will promote initiation complex formation on ribosomes from other organelles such as chloroplasts. As indicated in Fig. 3A, this factor was as active on chloroplast 30 S ribosomal subunits as on its homologous mitochondrial ribosomes. In contrast, IF-2_{chl} had no activity on mitochondrial ribosomes (Fig. 3B). This latter observation is perhaps not too surprising since this chloroplast initiation factor is not even active on prokaryotic ribosomes (18). The observations made with IF-2_{mt} are similar to those made with EF-G_{mt}. Both of these factors are active on *E. coli* ribosomes while their prokaryotic counterparts are not active on mitochondrial ribosomes (4, 10).

The initiation of translation in both prokaryotes and mitochondria occurs with formylmethionyl-tRNA. Animal mitochondria appear to have a single tRNA^{Met} gene. The tRNA^{Met} encoded by this gene bears a slightly closer relationship to the yeast initiator tRNA than to the *E. coli* initiator tRNA or to elongator tRNA^{Met} species. We routinely use yeast fMet-tRNA to assay IF-2_{mt}. In order to examine whether IF-2_{mt} could recognize the prokaryotic initiator tRNA and to examine the importance of the formyl group for the use of this tRNA for initiation, we tested the ability of IF-2_{mt} to promote initiation complex formation with fMet-tRNA and Met-tRNA from both yeast and *E. coli*. As indicated in Fig. 4, IF-2_{mt} was able to promote the binding of both *E. coli* and yeast fMet-tRNA to mitochondrial ribosomes. However, the binding of the nonformylated Met-tRNAs to the mitochon-

drial ribosomes was about 20-fold (Fig. 4A) and 50-fold (Fig. 4B) lower than that observed with their formylated counterparts. These results indicate that the mitochondrial factor, like *E. coli* IF-2 (11) shows a strong requirement for formylation of the initiator tRNA for initiation complex formation.

It has been reported that both *E. coli* IF-2 and the eukaryotic cytoplasmic factor eIF-2 can form binary complexes with GTP or GDP (12, 24, 25) although the bacterial factor binds this ligand much less tightly than does the eukaryotic cytoplasmic factor. We have examined the interaction of IF-2_{mt} with guanine nucleotides using an indirect assay based on the ability of a bound ligand to affect the temperature at which a protein is inactivated. In the presence of BSA, IF-2_{mt} was relatively stable at temperatures up to 27 °C. However, its activity declined rapidly thereafter and complete inactivation was observed after a 10-min incubation at 55 °C (data not shown). As indicated in Fig. 5, both GTP and GDP were effective in protecting IF-2_{mt} from heat inactivation. After 20 min at 42 °C, IF-2_{mt} retained only 30% of its activity. In contrast, IF-2_{mt} retained about 75% of its activity when incubated at this temperature in the presence of either GTP or GDP. The protection from thermal inactivation conferred by the binding of GTP or GDP to IF-2_{mt} probably results from a conformational change in the protein making it more compact or by the direct protection of labile asparagine or glutamine residues from deamination by the presence of the nucleotide (26).

Both *E. coli* IF-2 and IF-2_{chl} contain sulfhydryl groups that can be modified by *N*-ethylmaleimide resulting in the loss of activity (27, 28). Treatment of IF-2_{mt} with *N*-ethylmaleimide resulted in a loss of more than 95% of the activity of this factor (data not shown). When the *N*-ethylmaleimide treatment was carried out in the presence of either GTP or GDP, IF-2_{mt} maintained a significant portion of its activity (data not shown). The protection observed indicates that IF-2_{mt} interacts directly with guanine nucleotides and that nucleotide binding can potentially occur prior to the interaction of this factor with the ribosome.

As reported previously (10), GDP cannot replace GTP in promoting the binding of fMet-tRNA to mitochondrial ribosomes. However, the ability of GDP to protect IF-2_{mt} from heat or *N*-ethylmaleimide inactivation indicates that this factor can bind GDP. GDP is a potent inhibitor of the IF-2_{mt}-promoted binding of fMet-tRNA to mitochondrial ribosomes. A 1:1 ratio of GDP to GTP resulted in a 50% reduction in the

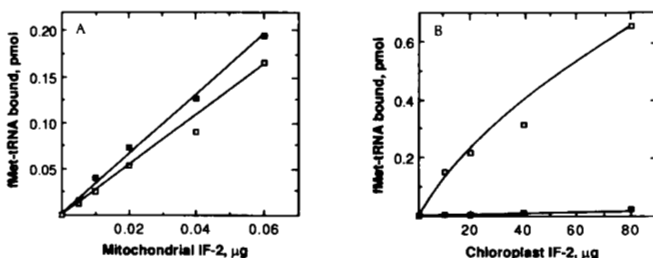


FIG. 3. Activities of IF-2_{mt} and IF-2_{chl} on mitochondrial and chloroplast ribosomes. Reaction mixtures were prepared as described under "Experimental Procedures." A, reaction mixtures contained the indicated amount of IF-2_{mt} and either 0.14 A₂₆₀ of mitochondrial ribosomes (■) or 0.05 A₂₆₀ of chloroplast 30 S ribosomal subunits (□). B, reaction mixtures contained the indicated amount of IF-2_{chl} and either 0.05 A₂₆₀ of chloroplast 30 S ribosomal subunits (□) or 0.14 A₂₆₀ of mitochondrial ribosomes (■).

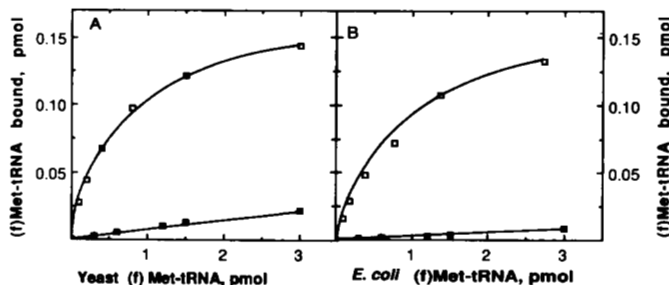


FIG. 4. The activity of IF-2_{mt} with formylated and nonformylated initiator Met-tRNA. All reaction mixtures contained 0.14 A₂₆₀ of mitochondrial ribosomes and 0.025 μg of IF-2_{mt}. A, reaction mixtures contained the indicated amount of either yeast fMet-tRNA (□) or Met-tRNA (■). B, reaction mixtures contained the indicated amount of either *E. coli* fMet-tRNA (□) or Met-tRNA (■).

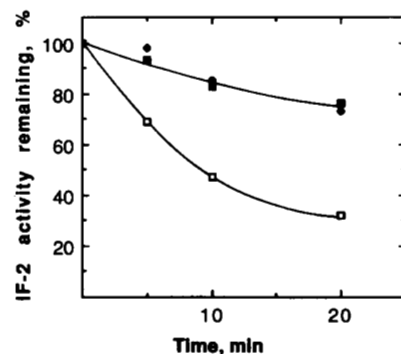


FIG. 5. Thermal inactivation of IF-2_{mt} in the presence of guanine nucleotides. Purified IF-2_{mt} (0.05 μg) was incubated for the indicated times at 42 °C alone (□), in the presence of 0.1 μM GTP (◆) or GDP (■). Aliquots were subsequently tested for activity in the fMet-tRNA binding assay as described under "Experimental Procedures." The value of 100% is defined as the amount of activity remaining in a sample left at -70 °C for the time course of the experiment and represents 0.11 pmol of fMet-tRNA bound.

amount of fMet-tRNA bound indicating that IF-2_{mt} has approximately equal affinities for these two nucleotides (data not shown). In contrast, GMP did not affect the activity on IF-2_{mt} suggesting that the mononucleotide does not bind to the mitochondrial initiation factor.

Both *E. coli* IF-2 and eukaryotic cytoplasmic eIF-2 are members of the class of proteins referred to as G-proteins. The requirement of IF-2_{mt} for GTP and the inhibition of its activity by GDP suggest that the organellar factor also falls into this class of proteins. We have examined the substituents on the GTP molecule important for its interaction with IF-2_{mt} by investigating the nucleotide specificity of this reaction. No fMet-tRNA binding could be observed when GTP was replaced by either UTP or CTP (data not shown) indicating that this factor is specific for purine nucleotides. Only a trace of activity was observed when GTP was replaced by ATP indicating that IF-2_{mt} requires a guanine nucleotide for activity (data not shown). However, deoxyGTP was as active as GTP in promoting fMet-tRNA binding to mitochondrial ribosomes (Fig. 6). These results indicate that the 2'-OH moiety on the ribose ring does not make important contacts with the protein. ITP which lacks the -NH₂ substituent at the 2 position of the base, was also somewhat active in promoting the binding of fMet-tRNA to ribosomes (Fig. 6). However, a 50–100-fold higher concentration of ITP was required compared to GTP indicating that IF-2_{mt} has some interaction with the -NH₂ group on the ring during ligand binding.

It has previously been reported that IF-2_{mt} may be functioning catalytically and is used several times during the course of the assay (10). This conclusion was based on the observation that the use of GTP led to about 3-fold more initiation complex formation than did the use of the nonhydrolyzable analog GMP-PNP. In the prokaryotic system, subunit joining occurs prior to GTP hydrolysis and the release of IF-2 occurs following 70 S initiation complex formation (11). In contrast, in the eukaryotic cytoplasmic system, eIF-5 promotes the hydrolysis of the GTP present in the ternary complex on the 40 S subunit (29). This interaction then allows the release of eIF-2 prior to the joining of the 60 S subunit. In this system, the presence of a nonhydrolyzable analog prevents the subunit joining reaction (30). In order to determine whether GTP hydrolysis is required before the 39 S mitochondrial subunit joins the 28 S initiation complex, we have examined the effects of nonhydrolyzable analogs on the formation of 28 S and 55 S initiation complexes using IF-2_{mt}. As indicated in Fig. 7, fMet-tRNA could be seen associated with both the 28 S subunit and the 55 S monosome in the

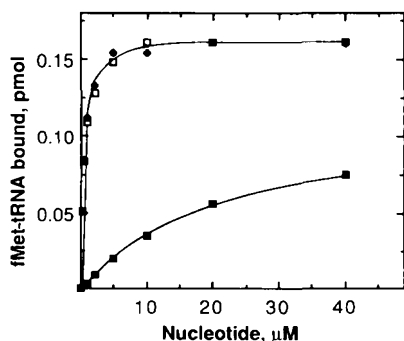


FIG. 6. Comparison of effect of GTP, dGTP, and ITP concentrations on the activity of IF-2_{mt}. Reaction mixtures contained 0.11 A₂₆₀ of mitochondrial ribosomes, 0.04 μg of purified IF-2_{mt}, and the indicated amount of GTP (□), dGTP (◆), or ITP (■). The ITP used for these experiments was prepared synthetically and was free of GTP contamination (Pharmacia).

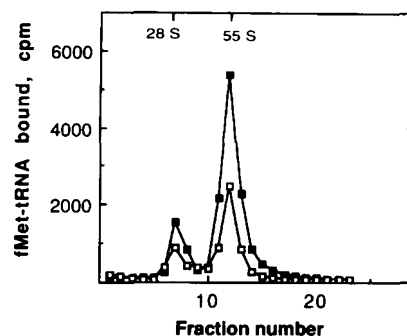


FIG. 7. The effect of GTP and GMP-PNP on the binding of [³⁵S]fMet-tRNA to mitochondrial ribosomes. Reaction mixtures were prepared and analyzed on sucrose density gradients as described under "Experimental Procedures." Mitochondrial ribosomes (0.42 A₂₆₀) and purified IF-2_{mt} (0.11 μg, 1.3 pmol) were incubated with either GTP (■; A) or GMP-PNP (□; B). Under these conditions, 0.86 pmol of fMet-tRNA was bound to ribosomes in the presence of GTP and 0.27 pmol were bound in the presence of GMP-PNP. The amount of fMet-tRNA present in each fraction was quantitated and the absorbance at 254 nm was used to determine the position of elution of the 28 S, 39 S, and 55 S particles.

presence of GTP. Furthermore, when GTP was replaced by the nonhydrolyzable analog GMP-PNP, both 28 S and 55 S complexes were again formed. This result indicates that the subunit joining step in mitochondrial protein synthesis does not require GTP hydrolysis and that, in this respect, the mitochondrial system resembles that of prokaryotes more closely than that found in the eukaryotic cytoplasm.

DISCUSSION

In the present work, we report the purification of the first animal mitochondrial translational initiation factor, IF-2_{mt}. The results presented here indicate that this factor resembles its prokaryotic counterpart in many general features. Like *E. coli* IF-2, the mitochondrial factor consists of a single polypeptide chain. This observation is in contrast to the eukaryotic cytoplasmic factor eIF-2 which consists of two or three different types of polypeptide chains (12). However, it should be noted that IF-2_{mt} is not closely related to *E. coli* IF-2 immunologically and that the bacterial factor is not active in promoting fMet-tRNA binding to animal mitochondrial ribosomes (10).

One of the most intriguing questions concerning the mechanism of protein biosynthesis in animal mitochondria lies in the observation that there is a single gene for tRNA^{Met} in the genome of this organelle (1). This gene may give rise to both fMet-tRNA for use in initiation and to Met-tRNA required for interaction with EF-Tu·Ts_{mt} during the elongation cycle. Alternatively, it is possible that one of the tRNA^{Met} species is imported from the cytoplasm for use in mitochondrial translation. The import of tRNAs into mitochondria occurs in several organisms (31, 32), and the import of RNA has been documented for animal mitochondria (33). Previous results (34) have demonstrated that initiation in animal mitochondria occurs with formyl methionine and the observations reported here clearly indicate that IF-2_{mt} requires the presence of the formyl group for initiation complex formation. Studies on EF-Tu·Ts_{mt} have shown that this elongation factor strongly discriminates against the initiator tRNA² indicating that some mechanism must be present in the mitochondria to ensure that tRNAs equivalent to the traditional initiator and elongator species are available to the translational ma-

² V. Worix, unpublished observations.

chinery. It should be noted that the strict requirement of IF-2_{mt} for the presence of a formyl group is again reminiscent of the bacterial initiation factor (7). Cytoplasmic eIF-2 will interact quite well with both Met-tRNA and fMet-tRNA in keeping with the use of the unformylated derivative for initiation in the cytoplasmic system (7).

Initiation complex formation promoted by IF-2_{mt} clearly requires the presence of GTP and the protection of this factor from heat and *N*-ethylmaleimide inactivation by guanine nucleotides indicates that IF-2_{mt} interacts with both GTP and GDP. A similar observation has been made for *E. coli* IF-2 (25) and eIF-2 (35). These initiation factors appear to belong in the broad class of guanine nucleotide binding proteins referred to as the G-proteins. However, there may be significant differences between IF-2 and eIF-2 in their interactions with guanine nucleotides. An examination of the sequence of *E. coli* IF-2 reveals that this factor contains the conserved basic consensus sequences found in most G-proteins (36). However, eIF-2 appears to have the nucleotide binding domain distributed between at least two subunits, and this factor may interact with nucleotides somewhat differently than other G-proteins (37). The results presented here indicate that deoxyGTP shows the same ability as GTP in promoting the binding of fMet-tRNA to mitochondrial ribosomes. This observation suggests that the 2'-hydroxyl group in ribose ring is not important for nucleotide binding to IF-2_{mt}. A similar observation has been made with *E. coli* IF-2 (25) and several other G-proteins including eIF-2 (38–40). None of the pyrimidine nucleotides tested in the fMet-tRNA binding was able to promote the formation of initiation complexes suggesting the functional groups of the pyrimidine ring do not match to the binding site in the active center of IF-2_{mt}. IF-2_{mt} showed some activity with ITP although it appears to have a lower affinity for this nucleotide. ITP differs from GTP only at the 2 position where it has a hydrogen atom instead of the amino group. The observation that IF-2_{mt} has a significant amount of activity with ITP suggests that only a weak H-bond interaction ($\Delta H \approx 2$ kcal/mol) occurs between IF-2_{mt} and the 2-NH₂ group of GTP. Proton NMR analysis suggests that *E. coli* IF-2 requires either a proton donor or acceptor at the C-2 position of the purine ring (25). The relative importance of the substituent at the 2-position of the purine ring appears to vary somewhat from one G-protein to another, but most of them show a preference for the presence of a H-bond donor in this position (41).

Work is in progress to explore additional properties of this factor and to seek additional factors that are likely to be involved in the initiation of protein synthesis in animal mitochondria.

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